Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Antitumor and Immunomodulation of Total Flavonoids from the Green Peep of *Juglans regia* L. in Mouse Forestomach Carcinoma Gastric Cancer-Bearing Mice

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Submitted: 02-Feb-2021 Revised: 15-Dec-2021 Accepted: 25-Jan-2022 Published: 07-Jul-2022

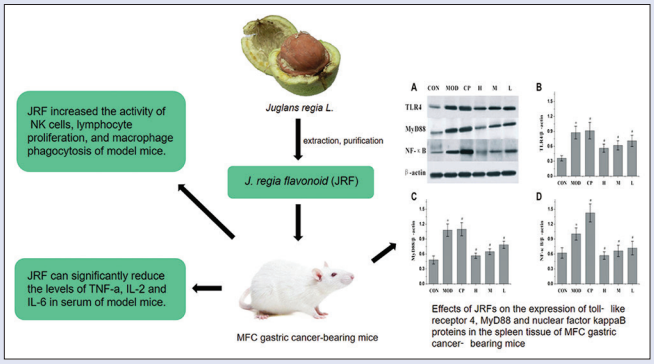
ABSTRACT

Background: Gastric cancer is a malignant tumor of the digestive tract. It is the fifth most common cancer and the third leading cause of cancer death across the world. In this study, we investigated the antitumor and immunomodulatory effects of total flavonoids from the green peel of *Juglans regia* L.(JRFs) in Mouse Forestomach Carcinoma (MFC) gastric cancer-bearing mice. **Methods:** In this study, an MFC gastric cancer-bearing mouse model was established. The killing activity of natural killer (NK) cells, the proliferation of lymphocytes, and the phagocytosis of macrophages of mice were observed by lactate dehydrogenase release assay and MTT assay. The levels of tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and IL-6 in the serum of mice were detected by enzyme-linked immunosorbent assay, and the mRNA and protein expressions of Toll-Like Receptor 4 (TLR4), MyD88, and nuclear factor kappaB (NF-κB) in the spleen tissue were detected by real-time polymerase chain reaction and Western blot analysis, respectively. **Results:** Compared with the model group, the tumor weight of mice in the high-, medium-, and low-dose JRF groups was significantly decreased, whereas the spleen index, the activity of NK cells, the proliferation of lymphocytes, and the phagocytosis of macrophages were significantly increased. There was no significant difference in the level of TNF-α, IL-2, and IL-6 in the serum of mice between the model group and the low-dose JRFs group, whereas the levels of TNF-α, IL-2, and IL-6 in the serum of mice in the high-and medium-dose JRF groups were significantly higher than those in the model group. The mRNA and protein expressions of TLR4, MyD88, and NF-κB in the spleen tissue of mice in the high-, medium-, and low-dose JRF groups were significantly lower than those in the model group. **Conclusion:** JRFs can inhibit the growth of MFC gastric cancer in mice and the blocked activation of TLR4/NF-κB signaling pathway may be the potential mechanism of the antitumor and immunomodulatory effects of JRFs in MFC gastric cancer-bearing mice.

Key words: Antitumor, flavonoids, immunomodulation, *Juglans regia L.*, mouse forestomach carcinoma gastric cancer-bearing mice, toll-like receptor 4/nuclear factor kappa B signaling pathway

SUMMARY

- The green peel of *Juglans regia* L.(walnut) immature fruit has a wide range of pharmacological effects, with a long history in treating malignant tumor in traditional Chinese medicine. Flavonoids are the primary constituents of the green peel of *J. regia* and are also the main active components. The results of this study indicate that JRF inhibits the growth of Mouse Forestomach Carcinoma (MFC) gastric cancer in mice and blocked the activation of Toll-Like Receptor 4/Nuclear factor-kappaB signaling pathway bringing about a potential antitumor and immunomodulatory effect in MFC gastric cancer-bearing mice.



Abbreviations used: JRFs: *Juglans regia* flavonoids; MFC: Mouse Forestomach Carcinoma; LDH: Lactate dehydrogenase; NK: Natural killer cell; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNF-α: Tumor necrosis factor-α; IL-2: Interleukin-2; IL-6: Interleukin-6; ELISA: Enzyme-linked immunosorbent assay; TLR4: Toll-Like Receptor 4; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor-kappaB; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; FBS: Fetal bovine serum; LSM: Lymphocyte separation medium; PBS: Phosphate buffer saline.

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DOI: 10.4103/pm.pm_54_21

Access this article online
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INTRODUCTION

Gastric cancer is the fifth most common cancer and the third leading cause of cancer deaths around the world.[1,2] A malignant tumor of the digestive tract, with the characteristics of high mortality, rapid development, and occult onset, has been considered a major disease that seriously endangers people's health.[3-5] An early diagnosis rate, surgical resection rate, and a 5-year survival rate of gastric cancer are relatively low, and most of the diagnosis is possible when the patients are in their middle and late stages of cancer.[6-8] In addition to surgery,

Cite this article as: Wei B, Zhu H, Wang K, Liu S, Dai Y, Liu D, *et al.* Antitumor and immunomodulation of total flavonoids from the green peep of *Juglans regia* L. in mouse forestomach carcinoma gastric cancer-bearing mice. Phcog Mag 2022;18:328-33.

the main treatment methods for gastric cancer include immunotherapy, radiotherapy, and chemotherapy, but the immunosuppression, tumor drug resistance, and serious drug adverse reactions can lead to an unsatisfactory therapeutic effect, which is also an important cause of death.[9-12] Traditional Chinese medicine has a long history in treating cancer, in particular, many Chinese herbal medicines have shown a unique effect in the treatment of cancer. For example, *Ginseng* can effectively alleviate the weakness caused by cancers and their treatment.[13] Grape, soybean, green tea, garlic, olive, and pomegranate can effectively prevent and treat colon cancer, and turmeric, ginger, and *Ashwagandha* have good preventive and therapeutic effects on gastric and hepatic cancers.[14-16]

In recent years, compounds extracted from traditional Chinese medicines, with an antitumor activity against gastric cancer, have attracted more and more attention.[17-19] *Juglans regia* L.(walnut) is a plant of Juglandaceae. In traditional Chinese medicine, the green peel of its immature fruit is called Qinglongyi and is used in clearing away heat and toxin, expelling wind and tinea, and relieving pain and dysentery.[20] Modern pharmacological studies have shown that the green peel of *J. regia* has a wide range of pharmacological effects, including anti-inflammatory,[21] analgesic,[22] antibacterial,[23] antioxidant,[24,25] and antitumor effects,[26,27] There are complex chemical constituents in the peel of *J. regia*, including naphthoquinones, glycosides, polysaccharides, flavonoids, diarylheptanoid, terpenoids, organic acids, and tannins.[28,29] Among the aforementioned compounds, flavonoids are the main active components that have attracted greater attention among researchers. So far, there are studies that have explored the therapeutic effect of green peel of *J. regia* against hepatic cancer[30] and colon cancer;[31] however, to the best of our knowledge, there is scarce information regarding the effect of *J. regia* peel flavonoids in the treatment of gastric cancer. Therefore, in this study, an Mouse Forestomach Carcinoma (MFC) gastric cancer-bearing mouse model was established and used to study on the *in vivo* antitumor and immunomodulatory effects of *J. regia* peel flavonoids, which was expected to provide a new feasible scheme for the treatment of gastric cancer in the future.

Experimental methods

Materials

Animals and cells

Fifty one-month-old SPF-grade BALB/c mice (half male and half female) weighing around 20 ± 2 g were purchased from Beijing Weitong Lihua Company (Beijing, China). The mice were raised in separate cages with a humidity of 45%–55% at a temperature of 20°C–25°C. The food and water were provided freely. MFC gastric cancer cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China).

Drugs and reagents

The green peel from immature fruit of *J. regia* was collected from Jiaohe City and identified by Jin Yinglan, the chief pharmacist of Jilin City Institute for Drug Control. Cyclophosphamide Tablets were obtained from Shanghai Hualian Pharmaceutical Co., Ltd., (Shanghai, China). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), and RPMI-1640 medium were obtained from Gibco (USA). Tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-6 detection kits were obtained from Nanjing Jiancheng Institute of Biology (Nanjing, China). RNA extraction kit, RR047A reverse transcription kit, and SYBR Premix Ex Taq TM II were obtained from Takara (Japan). Double distilled water was prepared in our laboratory.

Main instruments

SW-CJ-2FD ultra-clean worktable was obtained from Shanghai Bosun Biotechnology Co., Ltd., (Shanghai, China). BPN-RHP/RWP CO_2 incubator was obtained from Shanghai Yiheng Instrument Co., Ltd., (Shanghai China). ELx800TM microplate reader was obtained from BioTek (USA). Next, 760CRT UV visible spectrophotometer was obtained from Shanghai Jingke Instrument Co., Ltd., (Shanghai, China). Finally, CFX96 fluorescent quantitative PCR was obtained from Bio-Rad (USA).

METHODS

Extraction, purification, and determination of total flavonoids

The green peel of *J. regia* was dried, crushed, and sieved through a 40-mesh sieve. The powder was heated and refluxed twice with 10 times volume of 75% ethanol, 1 h and each time. The filtrates were combined, the solvent was recovered by decompression, and evaporated to dryness. The residue was dissolved in water, and the solution was added into a pretreated AB-8 macroporous resin column in several times and kept for 1 h. Then, the sample was eluted with 2 times volume of water. The eluent was discarded, and the sample was eluted with 1.5 times column volume of 75% ethanol, and the eluent was collected. The ethanol was recovered under decompression and the eluent was evaporated to dryness to obtain the flavonoids and named as *J. regia* flavonoid (JRF).

Modeling, grouping, and administration

MFC gastric cancer cells (1.5×10^6/mL) in logarithmic phase were inoculated under the armpit of BALB/c mice (0.2 mL per mouse). Then, 50 eligible mice were randomly divided into model group, cyclophosphamide group (20 $mg \cdot kg^{-1}$), high-dose JRF group (400 $mg \cdot kg^{-1}$), medium-dose JRF group (200 $mg \cdot kg^{-1}$), and low-dose JRF group (100 $mg \cdot kg^{-1}$), 10 mice in each group. For the control group, 10 healthy BALB/c mice were randomly selected. Based on the group they belong to, all mice were intragastrically administered once daily for up to 21 days. In the case of control group and model group, the animals were administered with an equal volume of normal saline.

Calculation of tumor inhibition rate and organ index

Briefly, 24 h after the last administration, the eyeballs were removed for collecting the blood of mice under the anesthesia with ether, and the serum was separated by centrifuging the blood and frozen until further use. Then, the mice were sacrificed through cervical dislocation, and the tumor mass, spleen, and thymus of mice were removed under aseptic conditions and weighed for the calculation of tumor inhibition rate and organ indexes according to the following equations.

Tumor inhibition rate = ([tumor weight in model group-tumor weight in drug-treated group]/tumor weight in model group) × 100%

Organ index = organ mass/body mass

Activity of natural killer cell

The mice were sacrificed through cervical dislocation, and the spleen was removed under aseptic conditions. The spleen was weighed and the splenic lymphocytes were separated, and the cell count was adjusted to 2×10^6/mL. Subsequently, 50 μL of YAC-1 cell solution (target cells) adjusted to the concentration of 1×10^5/mL were put into 96-well cell culture plates. To the culture plates, 50 μL of splenic lymphocyte suspension (effector cells) was added, and in addition, simple target cell and simple effector cell wells were set up, respectively. The cells were

cultured for 72 h under normal conditions. MTT method[32] was used to measure the absorbance at 429 nm for testing the killing activity of NK cells according to the following equation.

NK cell killing activity = $(1 - (A_s - A_0)/A_1) \times 100\%$.

Where A_s represents the absorbance value of the experimental wells, A_0 represents the absorbance value of effector cell wells, and A_1 represents the absorbance value of target cell wells.

Splenic lymphocyte proliferation test

The mouse spleen cell suspension was prepared according to the method used in section 1.2.4, and the cell count was adjusted to 2×10^6/mL. Subsequently, 100 μL of splenic lymphocyte suspension was added into 96-well cell culture plates. To this, 100 μL of concanavalin A was added, and the cells were cultured under normal conditions for 48 h. The absorbance was read at 450 nm.

Peritoneal macrophage phagocytosis function test

After the mice were sacrificed, the peritoneal cavity of the mice was lavaged with phosphate-buffered saline (PBS). The lavage fluid was collected, and the cells were centrifuged. The cell counts were added adjusted to 2×10^6/mL with DMEM medium, and 150 μL of this peritoneal macrophage suspension was added into 96-well cell culture plates. The cells were grown for 4 h under normal conditions. Then, the nonadherent cells were carefully sucked out and discarded, the medium was replaced by a fresh one. The cells were continued to be cultured for 12 h, and the medium was added with 50 μL of 3 mg/mL neutral red solution. After the medium was mixed well, the cells were cultured for 4 h, and then rinsed thrice with PBS. Subsequently, 150 μL of the cell lysis buffer was added to each well, and the absorbance was read at 540 nm using a microplate reader.

Detection of serum tumor necrosis factor-α, interleukin-2, and interleukin-6 levels

Briefly, 200 μL of the blood was collected by the enucleation of eyeballs, and 100 μL of the blood was used for the centrifugation to collect the serum. The levels of TNF-α, IL-2, and IL-6 in the serum were detected by enzyme-linked immunosorbent assay (ELISA) according to the operation guide of the kit manuals.

Expression of toll-like receptor 4, MyD88, and nuclear factor kappaB mRNA in the spleen

The total RNA was extracted by Trizol reagent, and the mRNA was reverse-transcribed into cDNA by using a RR047A reverse transcription kit. The mRNA expression of toll-like receptor 4 (TLR4), MyD88, and NF-κB was detected by SYBR method. The relative expression of target genes was calculated by $2^{-\Delta\Delta Ct}$ method with β-actin as the internal reference. The primers were synthesized by Dalian Bioengineering Technology Co., Ltd. (Dalian, China). Table 1 shows the sequences.

Western blot analysis

For Western blot analysis, 90 mg of the spleen tissue were cut into pieces, to which 810 μL of the tissue protein lysis buffer was added and placed on ice for 1 h for the preparation of spleen tissue homogenate. The homogenate was centrifuged for 10 min (12000 rpm, 4°C) to separate the supernatant, and the protein concentration in the supernatant was determined by bicinchoninic acid method. The protein samples were boiled for 5–10 min for denaturation, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk for 2 h and then incubated with the primary antibodies of TLR4, MyD88, and NF-κB at 4°C overnight. Then, the membrane was washed thrice with 100 mL TBST for 10 min and then incubated with the secondary antibodies for 1 h at room temperature. ECL developer and ChampChemi Professional + automatic multicolor fluorescence and chemiluminescence gel imaging system (Saizhi Technology Co, Ltd., Beijing, China) were used to visualize the proteins and analyze their grays. The relative expression of proteins was expressed by target protein/GAPDH or phosphorylated protein/total protein.

Statistical analysis

The data were statistically analyzed, and the results are expressed as mean ± standard deviation. Analysis of variance was used to compare the differences. Dunnett's 3 method was used for the test of heterogeneity of variance and least square difference method was used for the homogeneity of variance. A value with $P \leq 0.05$ indicated a statistically significant difference.

RESULTS

Effect of JRF on mouse forestomach carcinoma gastric cancer-bearing mice

After the intragastric administration of JRF for 21 days, the tumor weight of mice in the model group increased significantly as compared with the control group mice ($P < 0.05$). Compared with the model group, the tumor weight of mice in the high-, medium-, and low-dose JRF groups decreased significantly ($P < 0.05$). The tumor inhibition rate for high-, medium-, and low-dose JRF groups were 39.86%, 32.87%, and 22.03%, respectively [Table 2].

Effects of JRF on organ indexes of mouse forestomach carcinoma gastric cancer-bearing mice

Compared with the control group, the spleen and thymus indexes of mice in the model group were significantly decreased ($P < 0.05$). Compared with the model group, the spleen index of mice in the high-, medium-, and low-dose JRF groups was significantly increased ($P < 0.05$). Compared

Table 1: Primer sequences

Name of genes	Primer sequences (5′-3′)	Length/bp
TLR4	Forward: CATGGATCAGAAACTCAGCAAAGTC	176
	Reverse: CATGCCATGCCTTGTCTTCA	
MyD88	Forward: TACAGGTGGCCAGAGTGGAA	155
	Reverse: GCAGTAGCAGATAAAGGCATCGAA	
NF-κB	Forward: CCAAAGAAGGACACGACAGAATC	173
	Reverse: GGCAGGCTATTGCTCATCACA	
β-actin	Forward: CATCCGTAAAGACCTCTATGCCAAC	168
	Reverse: ATGGAGCCACCGATCCACA	

TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappaB

Table 2: Inhibition of *Juglans regia* L. on mouse forestomach carcinoma gastric cancer in mice (mean±s, *n*=10)

Group	Dose/mg•kg^{-1}	Tumor/g	Tumor inhibition rate/percentage
Control	-	0	-
Model	-	2.86±0.52*	-
Cyclophosphamide	20	1.20±0.33#	58.04
High-dose JRFs	400	1.72±0.36#	39.86
Medium-dose JRFs	200	1.92±0.39#	32.87
Low-dose JRFs	100	2.23±0.42#	22.03

*$P<0.05$, compared with the control group; #$P<0.05$, compared with the model group. JRFs: *Juglans regia* L

with the model group, the thymus index in the high-and medium-dose JRF groups was significantly increased ($P < 0.05$) [Table 3].

Effects of JRF on the natural killer cell activity, lymphocyte proliferation, and macrophage phagocytosis in mouse forestomach carcinoma gastric cancer-bearing mice

Compared with the control group, the activity of NK cells, the proliferation of lymphocytes, and the phagocytosis of macrophages in the model group were significantly decreased ($P < 0.05$). Compared with the model group, the activity of NK cells, the proliferation of lymphocytes, and the phagocytosis of macrophages in the high-, medium-, and low-dose JRF groups were significantly increased ($P < 0.05$) [Table 4].

Effect of JRF on the serum tumor necrosis factor-α, interleukin-2, and interleukin-6 levels in mouse forestomach carcinoma gastric cancer-bearing mice

Compared with the control group, the serum levels of TNF-α, IL-2, and IL-6 in the model group were significantly decreased ($P < 0.05$). There was no significant change in the serum level of TNF-α and IL-6 between the low-dose JRF and the model groups ($P > 0.05$), and the serum levels of TNF-α, IL-2, and IL-6 in medium-and high-JRF groups were significantly increased compared with those in the model group ($P < 0.05$) [Table 5].

Effect of JRF on mRNA expression of toll-like receptor 4, MyD88, and nuclear factor kappa-B in the spleen tissue of tumor-bearing mice

On the post-administration day 21, compared with those in the control group, the mRNA expression of TLR4, MyD88, and NF-κB in the spleen tissue of MFC gastric cancer-bearing mice was significantly increased in the model group ($P < 0.05$). Compared with the model group, the mRNA expression of TLR4, MyD88, and NF-κB in the cyclophosphamide group was significantly increased ($P < 0.05$). In the case of high-, medium-, and low-dose JRF groups, the mRNA expression of TLR4, MyD88, and NF-κB was significantly decreased ($P < 0.05$) [Table 6].

Table 3: Effects of *Juglans regia* L. on organ indexes of mouse forestomach carcinoma gastric cancer-bearing mice (mean±s, *n*=10)

Group	Dose/ mg•kg^{-1}	Spleen index/ mg•g^{-1}	Thymus index/ mg•g^{-1}
Control	-	6.52±0.83	2.65±0.38
Model	-	5.13±0.72*	1.96±0.29*
Cyclophosphamide	20	4.45±0.63#	1.78±0.22
High-dose JRFs	400	6.35±0.76#	2.49±0.35#
Medium-dose JRFs	200	6.12±0.68#	2.35±0.33#
Low-dose JRFs	100	5.86±0.61#	2.13±0.27

*$P<0.05$, compared with the control group; #$P<0.05$, compared with the model group. JRFs: *Juglans regia* L

Effect of JRF on the protein expression of toll-like receptor 4, MyD88, and nuclear factor kappa-B in the spleen tissue of mouse forestomach carcinoma gastric cancer-bearing mice

As shown in Figure 1, compared with the control group, the protein expression of TLR4, MyD88, and NF-κB in the spleen tissue of mice in the model group was significantly increased ($P < 0.05$). Compared with the model group, the expression of TLR4, MyD88, and NF-κB proteins in the spleen tissue of mice in the cyclophosphamide group was significantly decreased ($P < 0.05$). Furthermore, the expression of TLR4, MyD88, and NF-κB proteins in the high-, medium-, and low-dose JRF groups was significantly decreased ($P < 0.05$).

DISCUSSION

Gastric cancer is a malignant tumor of the digestive tract. It has a high incidence rate and a low survival rate and is the fifth most common cancer and the third leading cause of cancer death across the world. The

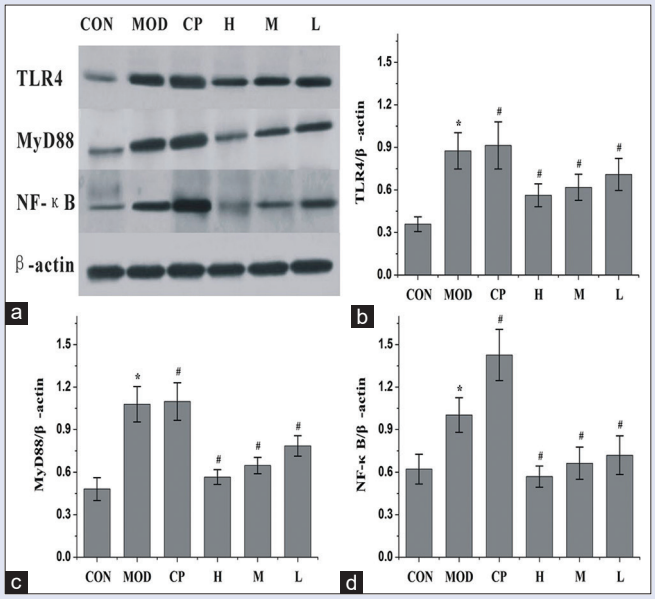

Figure 1: Effects of JRFs on the expression of toll-like receptor 4, MyD88 and nuclear factor kappaB proteins in the spleen tissue of MFC gastric cancer-bearing mice (mean ± *s*, *n* = 10) (a) Electrophoresis images of toll-like receptor 4, MyD88 and nuclear factor kappaB proteins detected by Western blot and (b-d) expressions of toll-like receptor 4, MyD88 and nuclear factor kappaB proteins by Western blot. CON: Blank control group; MOD: Model group; CP: Cyclophosphamide group; H: High-dose JRFs group; M: Medium-dose JRFs group; L: Low-dose JRFs group; *: $P < 0.05$, compared with the control group; #: $P < 0.05$, compared with the model group

Table 4: Effects of *Juglans regia* L. on the natural killer cell cytotoxicity, lymphocyte proliferation and macrophage phagocytosis in mouse forestomach carcinoma gastric cancer-bearing mice (mean±s, *n*=10)

Group	Dose/mg•kg^{-1}	NK cell killing activity/percentage	Lymphocyte proliferation	Macrophage phagocytosis
Control	-	69.25±8.81	0.83±0.09	0.95±0.13
Model	-	46.53±5.62*	0.49±0.06*	0.63±0.09*
Cyclophosphamide	20	36.18±4.33#	0.36±0.04#	0.42±0.07#
High-dose JRFs	400	65.86±8.69#	0.79±0.08#	0.89±0.11#
Medium-dose JRFs	200	61.21±7.86#	0.72±0.08#	0.85±0.12#
Low-dose JRFs	100	55.72±7.16#	0.64±0.05#	0.78±0.09#

*$P<0.05$, compared with the control group; #$P<0.05$, compared with the model group. JRFs: *Juglans regia* L.; NK: Natural killer

Table 5: Effects of *Juglans regia* L. on the serum tumor necrosis factor-α, interleukin-2 and interleukin-6 levels in mouse forestomach carcinoma gastric cancer-bearing mice (mean±s, *n*=10)

Group	Dose/mg•kg^{-1}	TNF-α/pg•ml^{-1}	IL-2/pg•ml^{-1}	IL-6/pg•ml^{-1}
Control	-	22.95±3.51	30.75±5.03	39.55±6.32
Model	-	18.56±2.26*	21.72±4.17*	27.36±5.92*
Cyclophosphamide	20	15.81±2.32#	17.67±3.96#	23.64±5.77#
High-dose JRFs	400	22.35±3.68#	28.91±5.28#	35.93±5.12#
Medium-dose JRFs	200	21.52±2.59#	27.22±5.53#	33.43±4.86#
Low-dose JRFs	100	19.82±2.36	25.83±4.69#	28.87±5.79

*P<0.05, compared with the control group; #P<0.05, compared with the model group. JRFs: *Juglans regia* L.; TNF-α: Tumor necrosis factor-α; IL: Interleukin

Table 6: Effects of *Juglans regia* L. on toll-like receptor 4, myeloid differentiation primary response 88 and nuclear factor kappaB micro ribonucleic acid expressions in the spleen tissue of mouse forestomach carcinoma gastric cancer-bearing mice (mean±s, *n*=10)

Group	Dose/ mg•kg^{-1}	TLR4	MyD88	NF-κB
Control	-	0.35±0.05	0.31±0.04	0.12±0.02
Model	-	0.56±0.08*	0.70±0.11*	0.32±0.04*
Cyclophosphamide	20	0.61±0.10#	0.78±0.13#	0.41±0.06#
High-dose JRFs	400	0.38±0.06#	0.30±0.03#	0.10±0.02#
Medium-dose JRFs	200	0.42±0.09#	0.34±0.04#	0.13±0.03#
Low-dose JRFs	100	0.49±0.08#	0.39±0.04#	0.19±0.03#

*P<0.05, compared with the control group; #P<0.05, compared with the model group. JRFs: *Juglans regia* L.; TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappaB; mRNA: Micro ribonucleic acid

5-year survival rate of patients with gastric cancer is only about 25%–30%.[1] Cyclophosphamide has been the drug of choice to treat gastric cancer, but it has serious side effects. In contrast, traditional Chinese medicines with a immunomodulatory function are characterized by mild adverse reactions, good antitumor activity and are not harmful to the immune function of the body.[33] In this study, the tumor weight of mice in the high-, medium-, and low-dose JRF groups was significantly reduced, and the tumor inhibition rates were 39.86%, 32.87%, and 22.03%, respectively. JRF increased the activity of NK cells, lymphocyte proliferation, and macrophage phagocytosis of model mice, which suggests that JRF can inhibit the growth of MFC gastric cancer in mice, and the effect can be attributed to its immunoregulation.

The immune system is the body's main tumor defense system. TNF-α, IL-2, and IL-6 are the main participants in the immune regulation in cancer.[34] TNF-α is not toxic to the normal cells, but it can kill tumor cells. It is the most potent antitumor cytokine, with the effects of enhancing the body's immune response, promoting tumor cell lysis, and inhibiting the proliferation of tumor cells.[35] IL-2, produced by activated T cells and with a strong antitumor effect, can induce the cytopoiesis of LAK cells, participate in the cellular immunity, enhance the activity of NK and CTL cells, and promote the effector cells to secrete TNF-α and IL-4. Therefore, it is known as the key protein and core regulatory factor in the immune system.[36] IL-6, produced by activated fibroblasts and T cells, can enhance the lytic function of NK cells, and the level of IL-6 and IL^{-2} can indirectly reflect the body's immune function and tumor progression.[37] The prognosis of patients with gastrointestinal cancers was positively correlated with the serum levels of IL-6 and IL^{-2}, and the detection of serum IL-6 and IL^{-2} levels was conducive to the prognosis and early diagnosis of tumors.[38] In this study, we confirmed that the serum levels of TNF-α, IL^{-2}, and IL-6 in mice in the medium-and high-dose JRF groups were significantly increased compared with those in the model group, indicating that JRF plays an immunomodulatory role by promoting the expression of immune factors and then inhibit the growth of tumor.

Recent studies have shown that the antitumor and immunomodulatory effects of many traditional Chinese medicines are related to TLRs signaling pathway, and among all TLRs signaling pathways, TLR4/NF-κB signaling pathway plays an important role in the antitumor and immunomodulatory effects of traditional Chinese medicines.[39] Therefore, in this study, we explored the effect of JRF on the expression of genes related to the TLR4/NF-κB signaling pathway. TLR4 can be expressed in all kinds of cells, and its activation can promote the production of immune factors.[40] MyD88 is the adaptor molecule in most signaling pathways of TLRs, and TLR4 can activate NF-κB through MyD88 and then regulate the secretion of immune factors.[41] NF-κB is an ideal regulatory target of immune signaling pathway, located in the downstream hub of TLRs signaling pathway.[42] Our results showed that the expression of TLR4, MyD88, and NF-κB mRNA in the spleen of mice in the high-, medium-and low-dose JRF groups was significantly decreased, indicating that JRF inhibited the TLR4/NF-κB signaling pathway and blocked the activation of TLR4/NF-κB signaling pathway. This mechanism of action needs to be further studied.

CONCLUSION

JRF can inhibit the growth of MFC gastric cancer in mice and block the activation of TLR4/NF-κB signaling pathway bringing about a potential antitumor and immunomodulatory effect in MFC gastric cancer-bearing mice.

Data availability statement

The data used to support the findings of this study are included within the article.

Ethical approval and informed consent

All animal experiments comply with the ARRIVE guidelines and followed the U. K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Acknowledgements

We wish to thank chief pharmacist Jin Yinglan for the identification of the green peel of immature fruit of *Juglans regia* L.

Financial support and sponsorship

This research was supported by the key medical and health innovation project of Jilin city (20190403007), Promotion project of clinical rational use of drugs of Department of Health of Jilin Province (Y202009), the department of education "13th five-year plan" science and technology project of Jilin Province (JJKH20200061KJ), the medical and health guidance plan project of Jilin city (201900982) and the Traditional Chinese medicine science and technology project of Jilin Province (2018110).

Conflicts of interest

The authors declare no competing interests.

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